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Phylogenetic Analysis of Two New Mitochondrial Genomes of *Singapora shinshana* and *Seriana bacilla* from the Karst Region of Southwest China

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Abstract: Leafhoppers have been identified as a serious threat to different plants. To explore the characteristics of mitogenomes and reveal the phylogenetic positions of two species in the Typhlocybinae, complete mitogenomes of Singapora shinshana and Seriana bacilla were sequenced and annotated for the first time with lengths of 15,402 bp and 15,383 bp, respectively. The two mitogenomes contained 13 PCGs, 22 tRNA genes and 2 rRNA genes. The genome content, gene order, nucleotide composition, codon usage and amino acid composition are similar to those of other typical mitogenomes of Typhlocybinae. All 13 PCGs started with ATN codons, except for atp8 (TTA) and nad5 (TTG). All tRNAs were folded into a typical cloverleaf secondary structure, except for tRNA-Ser1 and tRNA-Val. Moreover, phylogenetic trees were constructed and analyzed based on all the PCGs from 42 mitogenomes using maximum likelihood (ML) and Bayesian inference (BI) methods. The results supported that eleven subfamilies are all monophyletic groups, S. shinshana and S. bacilla are members of Erythroneurini, but S. shinshana and the genus Empoascanara have a very close relationship with ((((Empoascanara sipra+ Empoascanara wengangensis) + Empoascanara dwalata) + Empoascanara gracilis) + S. shinshana), and S. bacilla is closely related to the genus Mitjaevia ((Mitjaevia dworakowskae + Mitjaevia shibingensis) + S. bacilla). These results provide valuable information for future study of evolutionary relationships in Typhlocybinae.

Keywords: S. shinshana; S. bacilla; mitogenome; phylogenetic analysis; Karst

1. Introduction

Phytophagous piercing–sucking insects are insects that feed on the undersides of leaves and insert piercing–sucking mouthparts into the plant tissue to remove plant juices [1]. These insects are some of the main pests that feed on food crops, vegetables and ornamental plants [2]. Cicadellidae, the largest family of Hemiptera, is a type of insect harmful to cereals, vegetables, fruit trees and other trees [3]. *S. shinshana* and *S. bacilla* (Typhlocybinae, Cicadellidae, Hemiptera, Erythroneurini) cause significant damage to leaves of peach trees and potatoes by their sucking behavior, thus leading to yellow-whitish spots on the leaves [4,5]. This damage has a serious impact on the normal growth of agricultural and forestry crops and cause a decline in fruit yield.

The mitochondrial genome plays a crucial part in the phylogenetic and evolutionary analysis of insects. It is widely used to study genome structure and function, population genetic structure and phylogenetic relationships at various taxonomic levels [6–13]. Recently, it has also been used in the phylogenetic discussion of leafhoppers, including Deltocephalinae, Macropsinae, Megophthalminae, Idiocerinae, Iassinae, Coelidiinae, Ledrinae, Evacanthinae, Cicadellinae, Mileewinae and Typhlocybinae [9,14–19]. Guizhou is believed to be one of the most well-developed representative karst regions of south China.



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Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Many genera and species of Erythroneurini Young, 1952 were discovered in karst areas in Guizhou [20–23]. In recent years, the majority of studies have focused on the identification and recording of the genera *Singapora* and *Seriana* [24–26]. In addition, the seasonal phenology and damage caused by *S. shinshana* have attracted the attention of many experts [4]. However, the lack of mitogenome sequences for *S. shinshana* and *S. bacilla* has limited our understanding of their phylogenetic position within Erythroneurini.

In this study, we successfully sequenced and annotated the complete mitochondrial genome sequences of *S. shinshana* (GenBank accession no. OM048770.1) and *S. bacilla* (OM048922.1) from karst areas for the first time. The newly sequenced mitogenomes were compared with other available mitogenomes of related subfamilies/tribes to (1) analyze the structural and genomic features of the mitogenomes of *S. shinshana* and *S. bacilla*, (2) identify codon usage and (3) construct and elucidate comprehensive phylogenetic relationships (Table 1). The purpose of this work was to enrich the growing mitochondrial database for the Erythroneurini and clarify the phylogenetic position of the two species.

Table 1. List of sequences used to construct the phylogenetic tree.

Subfamily	Species	Accession Number	Reference
Ledrinae	Tituria sagittata	NC_051528.1	[27]
	Petalocephala eurglobata	MW018817.1	[28]
Evacanthinae	Evacanthus acuminatus	MK948205.1	[29]
	Evacanthus heimianus	MG813486.1	[30]
Mileewinae	Mileewa rufivena	MZ326689.1	[31]
	Mileewa albovittata	MK138358.1	[32]
	Eurhadina fusca	MZ983367.1	Direct submission
	Eurhadina dongwolensis	MZ457332.1	Direct submission
	Eurhadina jarrayi	MZ014455.1	[33]
	Agnesiella roxana	MZ457328.1	Direct submission
	Agnesiella kamala	MZ457327.1	Direct submission
	Eupteryx minuscula	MN910279.1	[34]
	Eupteryx gracilirama	MT594485.1	[35]
	Limassolla emmrichi	MW272458.1	[19]
Truchloauhingo	Limassolla lingchuanensis	NC_046037.1	[36]
турносубщае	<i>Limassolla</i> sp.	MT683892.1	[37]
	Mitjaevia shibingensis	MT981879.1	[16]
	Mitjaevia dworakowskae	MT981880.1	[16]
	Empoascanara wengangensis	MT445764.1	[17]
	Empoascanara sipra	NC_048516.1	[38]
	Empoascanara dwalata	MT350235.1	[39]
	Empoascanara gracilis	MT576649.1	[17]
	Singapora bacilla	OM048922.1	This study
	Seriana shinshana	OM048770.1	This study
	Homalodisca vitripennis	NC_006899.1	Direct submission
Cicadellinae	Cofana yasumatsui	NC_049087.1	[40]
	Cladolidia biungulata	NC_067788.1	[18]
Colidiinae	Cladolidia robusta	NC_067789.1	[18]
	Olidiana tongmaiensis	NC_057966.1	[18]
	Krisna concava	NC_046067.1	[8]
Iassinae	Krisna rufimarginata	NC_046068.1	[8]
	Batracomorphus lateprocessus	NC_045858.1	[8]
Hulicipao	Nacolus tuberculatus	NC_056923.1	[41]
Tryncinae	Kalasha nativa	NC_056922.1	[41]
Macropsinae	Oncopsis nigrofasciata	MG813492.1	[42]
Megophtthalinae	Durgades nigropicta	NC_035684.1	[6]
megopinnumme	Japanagallia spinosa	NC_035685.1	[6]

Subfamily	Species	Accession Number	Reference
	Roxasellana stellata	NC_050257.1	[15]
Deltocephalinae	Drabescus ineffectus	NC_050258.1	[15]
-	Reticuluma hamata	NC_051985.1	[43]
Outgroups	Cosmoscarta dorsimacula	NC_040115.1	[44]
(Cercopidae)	Callitettix versicolor	NC_020031.1	[45]

Table 1. Cont.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

The specimens were collected by a sweeping net in karst areas, and morphological terminology used in this work follows Dietrich (2005) and Song and Li (2013) [46,47]. Detailed collection information is shown in Table 2. The specimens were preserved in 95% ethanol and stored in the insect specimen storage room of Guizhou Normal University. The identified species were selected, and their head, wings and abdomen were removed. Genomic DNA was extracted from thorax muscle tissues and legs by employing a DNeasy Blood & Tissue kit (QIAGEN, Beijing, China). The tissues were ground and incubated at 56 °C for 6 h for complete lysis and total genomic DNA was eluted in 50 μ L of double distilled water (ddH₂O), and the remaining steps were performed according to the manufacturer's protocol. Genomic DNA was stored at -20 °C.

Table 2. The voucher information of the specimens used for mitochondrial genome sequencing in this study.

Species	Accession Number	Location	Collection Date
S. shinshana	GZNU-ELS-20190801	Huajiang, Guizhou (25°42′30″ N, 105°43′20″ E)	10 June 2019
S. bacilla	GZNU-ELS-20190601	Shibing, Guizhou (27°0'31.47″ N, 108°01'50.66″ E)	4 August 2019

2.2. Mitochondrial Genome Sequencing and Assembly

The complete mitochondrial genomes were sequenced at Berry Genomics (Beijing, China) using an Illumina Novaseq 6000 platform (Illumina, Alameda, CA, USA) with 150 bp paired-end reads. Firstly, the obtained sequence reads were filtered following Zhou et al. [48] and the residual high-quality reads were assembled by an iterative De Bruijin graph de novo assembler, the IDBA-UD toolkit, with a similarity threshold of 98% and *k* values of 40 and 160 bp [49]. The mitogenome was assembled by Geneious Prime 2021 v2021.1.1 using the clean paired reads with default parameters and the mitogenome of *Mitjaevia protuberanta* Song, Li et Xiong, 2011 (Hemiptera: Cicadellidae: Typhlocybinae) (GenBank accession number: NC_047465.1) as the reference [50], and then Geneious Prime software was used to manually map the clean readings to the obtained mitochondrial scaffolds to check the accuracy of the assembly.

2.3. Mitogenome Annotation and Sequence Analysis

The assembled mitogenome sequence was subsequently annotated using Geneious Prime and the mitogenome of *M. protuberanta* (GenBank accession number: NC_047465.1) as the reference. All tRNA genes were identified with the MITOS Web Server (http://mitos.bioinf.uni-leipzig.de/index.py, accessed on 12 December 2021) [51]. The annotated mitogenome sequences of *S. shinshana* and *S. bacilla* were deposited in GenBank under the accession numbers OM048770.1 and OM048922.1. The typical secondary structures for tRNAs were drawn with Adobe Illustrator 2021 in accordance with the MITOS predictions. The circular mitogenomic maps were visualized with the CGView server (http://stothard.afns.ualberta.ca/cgview_server/, accessed on 12 December 2021). The base composition,

codon usage and relative synonymous codon usage (RSCU) of all protein-coding genes (PCGs) were calculated using MEGA 7.0 [52]. Strand asymmetry was calculated using the following formulae: AT skew = (A - T)/(A + T) and GC-skew = (G - C)/(G + C) [53].

2.4. Phylogenetic Analyses

Forty species of Cicadellidae and two outgroups available on GenBank were selected to construct the phylogenetic tree (Table 1). Phylogenetic analyses were performed using the thirteen PCGs of the two species and other leafhopper species. Each PCG was aligned using MAFFT to perform protein alignment [54,55]. Gblocks version 0.91b was used to remove gaps and ambiguously aligned sites [52]. For phylogenetic analyses, the 13 PCG dataset was used to construct phylogenetic trees based on maximum likelihood (ML) and Bayesian inference (BI) using RaxML 8.0.2 [56] and MrBayes 3.2.6 [57], respectively. ML analysis was performed with 1000 rapid bootstrapping replicates using iqtree, and GTR+I+G was considered the best model. BI analysis was performed under the GTR+I+G nucleotide substitution model in MrBayes 3.2.7a with 4 chains and sampling of the chains every 1000 generations [58]. Then, 2 independent runs of 1,000,000 generations were applied.

3. Results and Discussion

3.1. Genome Organization and Composition

As with the reports of most leafhoppers, genome organization and composition were relatively conservative [8]. The complete mitogenomes of S. shinshana and S. bacilla are double-stranded plasmids with lengths of 15,204 bp and 15,383 bp (Figure 1), which contain 13 PCGs, 22 tRNA genes, 2 rRNA genes, and a control region (Table 3). However, the sequence lengths of *S. shinshana* and *S. bacilla* are different from of those of leafhoppers in complete mitochondrial genomes, based on the length of intergenic space and A+T-rich regions. There was no difference in the mitochondrial genome between the two species, and the content of A and T is higher than G and C. Twenty-three genes are located in the majority strand (J), whereas fourteen genes are encoded in the minority strand (N). Among them, the shortest intergenic space (1 bp) is located between tRNA-Ser2 and nad1, and the longest intergenic space (5 bp) is located between tRNA-Cys and tRNA-Tyr in S. shinshana. However, the shortest intergenic space sequence (1 bp) is located between atp6 and cox3, and the longest intergenic space sequence (11 bp) is located between tRNA-Gln and tRNA-Met in S. bacilla. Additionally, 13 PCG genes were found to overlap by a total of 37 bp and 35 bp in S. shinshana and S. bacilla, respectively (Table 3). The conserved 8 bp overlapping nucleotide sequence in the two species, located in tRNA-Typ and tRNA-Cys, is extremely common in Cicadellidae [6,18,42,59], whereas the other 8 bp overlapping nucleotide sequence between *nad6* and *cytb* from *S. shinshana* and the 10 bp overlapping nucleotide sequence from S. bacilla between tRNA-Ser2 and nad1 could be an accident.



Figure 1. Circular map of the mitochondrial genomes of S. shinshana and S. bacilla.

Gene	Posi	tion	Len	ıgth	Inter	genic	Start (Codon	Stop Codon		Strand
	SS	SB	SS	SB	SS	SB	SS	SB	SS	SB	
tRNA-Ile	1–63	1–64	63	64	0	0					J
tRNA-Gln	66–133	63-129	68	67	2	$^{-2}$					Ň
tRNA-Met	134-202	141-209	69	69	0	11					J
nad2	203-1174	210-1181	972	972	0	0	ATT	ATA	TAA	TAA	Ĵ
tRNA-Trp	1173-1240	1180-1248	68	69	-2	$^{-2}$					Ĵ
tRNA-Cys	1233-1293	1241-1301	61	61	-8	-8					Ň
tRNA-Tyr	1299-1361	1311-1378	63	68	5	9					Ν
cox1	1364-2902	1381-2916	1539	1536	2	2	ATG	ATG	TAA	TAA	J
tRNA-Leu1	2898-2964	2920-2985	67	66	-5	3					Ĵ
cox2	2965-3643	2986-3664	679	679	0	0	ATA	ATT	Т	Т	Ĵ
tRNA-Lys	3644-3714	3665-3735	71	71	0	0					Ĵ
tRNA-Asp	3714-3775	3736-3801	62	66	$^{-1}$	0					Ĵ
atp8	3774-3926	3801-3953	153	153	$^{-2}$	-1	TTG	TTG	TAA	TAA	Ĵ
atp6	3923-4573	3950-4600	651	651	-4	-4	ATA	ATA	TAA	TAA	J
cox3	4574-5353	4602-5381	780	780	0	1	ATG	ATG	TAA	TAA	Ĵ
tRNA-Gly	5356-5417	5387-5448	62	62	2	5					Ĵ
nad3	5418-5771	5449-5802	354	354	0	0	ATA	ATT	TAG	TAA	Ĵ
tRNA-Ala	5770-5832	5805-5865	63	61	$^{-2}$	2					J
tRNA-Arg	5835-5894	5866-5924	60	59	2	0					Ĵ
tRNA-Asn	5894-5958	5925-5990	65	66	$^{-1}$	0					J
tRNA-Ser1	5958-6024	5990-6057	67	68	$^{-1}$	-1					J
tRNA-Glu	6024-6089	6061-6125	66	65	$^{-1}$	3					J
tRNA-Phe	6090-6153	6124-6188	64	65	0	$^{-2}$					Ň
nad5	6154-7827	6189-7863	1674	1675	0	0	ATT	ATT	TAA	Т	Ν
tRNA-His	7828-7893	7861-7924	66	64	0	-3					Ν
nad4	7894-9201	7925-9247	1308	1323	0	0	ATA	ATA	TAA	TAA	Ν
nad4L	9201-9476	9247-9531	276	285	$^{-1}$	-1	ATG	ATA	TAA	TAA	Ν
tRNA-Thr	9479-9543	9536-9600	65	65	2	4					J
tRNA-Pro	9544-9609	9601–9666	65	66	0	0					Ν
nad6	9612-10,103	9669-10,154	492	486	2	2	ATT	ATT	TAA	TAA	J
cytb	10,096–11,232	10,159–11,295	1137	1137	-8	4	ATG	ATG	TAA	TAA	J
tRNA-Ser2	11,232–11,295	11,295–11,358	64	64	$^{-1}$	-1					J
nad1	11,297–12,227	11,349–12,290	931	942	1	-10	ATT	ATT	Т	TAA	Ν
tRNA-Leu2	12,228–12,292	12,291–12,355	65	65	0	0					Ν
16S	12,293-13,478	12,356-13,540	1186	1185	0	0					Ν
tRNA-Val	13,479–13,542	13,541–13,607	64	67	0	0					Ν
12 <i>S</i>	13,543–14,278	13,608–14,338	736	731	0	0					Ν
D-loop	14,279–15,204	14,339–15,383	926	1045	0	0					J

Table 3. Annotations of the mitogenomes of S. shinshana (SS) and S. bacilla (SB).

The total nucleotide content per base of *S. shinshana* and *S. bacilla* mitogenomes were, respectively, A (42.9%, 40.8%), T (36.1%, 35.3), G (9.4%, 10.8%) and C (11.7%, 13.0%). Like most insects [60], they had a strong A+T bias, and the content of A+T (78.9%, 76.1%) was significantly higher than that of G+C (21.1%, 23.9%) (Table 4). The overall positive AT skews (0.09, 0.07) and negative GC skews (-0.11, -0.09) indicated that A nucleotide content was higher than T content, and C content was higher than G nucleotide content. The AT skew and GC skew values of the two species are consistent with those reported earlier in leafhoppers [17].

S. shinshana	Size (bp)	A%	C%	G%	Τ%	A+T%	AT Skew	GC Skew
Full genome	15,204	42.9	11.7	9.4	36.1	78.9	0.09	-0.11
PCGs	10,946	42.7	12.8	10.5	34.0	76.7	0.11	-0.53
1st codon position	3649	45.9	11.8	10.4	32.0	77.9	0.18	-0.06
2nd codon position	3649	40.1	12.9	11.5	35.0	75.1	0.07	-0.06
3rd codon position	3648	42.1	13.7	9.6	35	77.1	0.09	-0.18
tRNA	1436	42.0	11.1	9.6	37.3	79.3	0.06	-0.07
rRNA	1922	47.6	10.8	6.6	35.1	82.7	0.15	-0.24
D-loop	926	36.5	0.9	2.5	60.2	96.7	-0.24	0.48
S. bacilla								
Full genome	15,383	40.8	13.0	10.8	35.3	76.1	0.07	-0.09
PCGs	10,973	39.4	14.5	12.4	33.7	73.1	0.08	-0.08
1st codon position	3658	40.3	15.1	14.4	30.0	70.3	0.15	-0.02
2nd codon position	3658	36.3	16.4	11.8	35.0	71.3	0.02	-0.16
3rd codon position	3657	41.5	11.8	11.2	36	77.5	0.07	-0.03
tRNA	1438	39.2	12.0	11.0	37.8	77.0	0.02	-0.04
rRNA	1916	48.1	11.7	6.5	33.7	81.8	0.18	-0.29
D-loop	926	36.5	0.9	2.5	60.2	96.7	-0.25	0.47

Table 4. Nucleotide compositions, AT skew and GC skew in different regions of *S. shinshana* and *S. bacilla*.

3.2. Protein-Coding Genes and Codon Usage

The total lengths of all the PCGs of *S. shinshana* and *S. bacilla* were 11,400 bp and 10,973 bp, accounting for 72.0% and 71.3% of the entire mitogenome, respectively. The order of the PCGs is shown in Figure 2. The longest PCG is *nad5* with 1674 bp and 1675 bp, respectively, and *atp8* is the shortest PCG with 153 bp. This phenomenon also occurs in other species of Cicadellidae [18]. Twelve PCGs of the *S. shinshana* and *S. bacilla* mitogenomes were initiated with the start codon ATN (ATG, ATA, ATT), except *atp8* (TTG). TAA is the most frequent stop codon, but in *cox2* and *nad1* in *S. shinshana* and *cox2* and *nad5* in *S. bacilla*, a single T is used as an incomplete stop codon, which is converted into a complete TAA codon through adding of a polyadenylated tail at the 3' end [55]. Only four genes, including *nad5*, *nad4*, *nad4L* and *nad1*, are located on the N-strand, and the remaining genes, including *cox1*, *cox2*, *cox3*, *atp8*, *atp6*, *nad2*, *nad6*, and *cytb*, are located on the J-strand.



Figure 2. Occurrence and sequence of all the PCGs from S. shinshana and S. bacilla.

The codon usage analysis of *S. shinshana* showed that the most frequently used codon was AAU-Asn (298), followed by AAA-Lys (250), AUU-Ile (250), AUA-Met (243) and UUA-Leu2 (212). However, UUA-Leu2 had the highest RSCU value (3.27) (Table 4 and Figure 3). In a previous study, the most frequently used codon was AUA, and this result is inconsistent with a previous study for Cicadellidae [18]. However, with regard to the codon usage analysis of *S. bacilla*, the most frequently used codons decreased in the following order: AAA-Lys (261), AUU-Ile (255), AAU-Asn (210), UUA-Leu2 (205) and AUA-Met (201) (Table 5). Codon usage analysis revealed that adenine and cytosine are usually located at the third codon. The most frequently used codons end with A or U, therefore, the content of AT is higher than that of GC in PCGs. More generally, this contributes to the AT content of the whole mitogenome.



Figure 3. Relative synonymous codon usage (RSCU) and codon number of *S. shinshana* and *S. bacilla*.

Amino Acid	Codon	Count/RSCU A				Amino Acid	Codon	Count/RSCU			
		S. shii	ıshana	S. bi	ncilla			S. shir	ishana	S. ba	acilla
Phe	UUU	145	1.42	167	1.54	Tyr	UAU	181	1.52	114	1.42
	UUC	59	0.58	50	0.46		UAC	57	0.48	47	0.58
Leu2	UUA	212	3.27	205	2.96	His	CAU	42	1.2	59	1.46
	UUG	28	0.43	52	0.75		CAC	28	0.8	22	0.54
Leu1	CUU	42	0.65	59	0.85	Gln	CAA	70	1.75	80	1.5
	CUC	18	0.28	8	0.12		CAG	10	0.25	27	0.5
	CUA	72	1.11	65	0.94	Asn	AAU	298	1.44	210	1.48
	CUG	17	0.26	26	0.38		AAC	116	0.56	74	0.52
Ile	AUU	250	1.58	255	1.74	Lys	AAA	250	1.53	261	1.65
	AUC	67	0.42	38	0.26		AAG	76	0.47	55	0.35
Met	AUA	243	1.71	201	1.64	Asp	GAU	35	1.43	53	1.39
	AUG	42	0.29	44	0.36		GAC	14	0.57	23	0.61
Val	GUU	36	1.45	51	1.62	Gln	GAA	61	1.56	98	1.69
	GUC	11	0.44	8	0.25		GAG	17	0.44	18	0.31
	GUA	41	1.66	52	1.65	Cys	UGU	35	1.35	29	1.38
	GUG	11	0.44	15	0.48		UGC	17	0.65	13	0.62
Ser2	UCU	37	0.93	43	1.19	Trp	UGA	46	1.3	55	1.31
	UCC	17	0.43	18	0.5		UGG	25	0.7	29	0.69
	UCA	92	2.31	88	2.43	Arg	CGU	10	1.08	11	0.83
	UCG	5	0.13	8	0.22	-	CGC	4	0.43	9	0.68

Table 5. Codon and relative synonymous codon usage (RSCU) of 13 PCGs of *S. shinshana* and *S. bacilla*.

Amino Acid	Codon	Count/RSCU				Amino Acid	Codon	Count/RSCU			
		S. shi	nshana	S. bacilla				S. shinshana		S. ba	icilla
Pro	CCU	25	1.06	55	1.42		CGA	19	2.05	25	1.89
	CCC	18	0.77	36	0.93		CGG	4	0.43	8	0.6
	CCA	48	2.04	49	1.26	Ser1	AGU	41	1.03	42	1.16
	CCG	3	0.13	15	0.39		AGC	23	0.58	20	0.55
Thr	ACU	43	1.04	79	1.57		AGA	71	1.79	50	1.38
	ACC	40	0.96	40	0.8		AGG	32	0.81	21	0.58
	ACA	70	1.69	75	1.49	Gly	GGU	26	1.12	39	1.3
	ACG	13	0.31	7	0.14	-	GGC	4	0.17	8	0.27
Ala	GCU	15	1.02	33	1.52		GGA	37	1.59	28	0.93
	GCC	8	0.54	18	0.83		GGG	26	1.12	45	1.5
	GCA	35	0.37	30	1.38	*	UAA	168	1.61	155	1.38
	GCG	1	0.07	6	0.28		UAG	41	0.39	33	0.62

Table 5. Cont.

* stands for stop codon.

3.3. Ribosomal and Transfer RNA Genes

The 16S rRNA gene contained 1186 bp and 1185 bp located between tRNA-*Leu2* and tRNA-*Val* in *S. shinshana* and *S. bacilla*, and the 12S rRNA gene comprised 736 bp and 731 bp situated between tRNA-*Val* and the D-loop in *S. shinshana* and *S. bacilla*. The A+T contents of 16S rRNA and 12S rRNA were 83.4%, 85.4% and 81.4%, 80.0%, respectively. Twenty-two tRNA genes from the two species ranged from 61 and 71 bp in both species. Among the 22 tRNA genes, 14 genes are located on the J-strand and 8 genes on the N-strand. This arrangement of tRNA-*Ile* and tRNA-*Gln* have been observed in the mitogenome of other subfamilies of Cicadellidae [6,8]. In insects, most tRNA genes are folded into the typical cloverleaf secondary structure, however, tRNA-*Ser1* lacks a dihydrouridine (DHU) arm [61] (Figure 4). Usually, the canonical Watson–Crick base pairings (G–U) are found in the DHU arms. The A+T contents in tRNA of *S. shinshana* and *S. bacilla* are 79.3% and 77.0% with a positive AT skew (0.06, 0.02) and a negative GC skew (-0.07, -0.04) (Table 4).

3.4. A+T-Rich Region

The mitochondrial A+T-rich region plays an important role in the initiation and regulation of insect replication and transcription in insects [62]. The A+T-rich region is located between $12S \ rRNA$ and tRNA-*lle* in the mitogenome with a total length of 926 bp and 1045 bp (Table 3) in *S. shinshana* and *S. bacilla*, respectively. The A+T content is 96.7% with both a negative AT skew (-0.24, -0.25) and a GC skew (0.48, 0.47) (Table 4).

3.5. Phylogenetic Relationship

The two phylogenetic relationships were constructed using the 13 PCGs by BI and ML methods and then they were merged into one phylogenetic tree. The results indicated that eleven subfamilies are all monophyletic groups, which is consistent with previous reports on phylogenetic research [18,43]. The main topology was as follows: (((Ledrinae + (Evacanthinae + Mileewinae) + (Typhlocybinae + Cicadellinae) + ((Hylicinae + (Coelidiinae + Iassinae)) + (Macropsnae + Megophthalmina))) + Deltocephalina). This result is consistent with phylogenetic relationships of previous studies [18], but different from reports by other researchers, whose results indicate phylogenetic relationships as follows: (Cicadellinae + (Deltocephalinae + (Typhlocybinae + Eurymelinae))) [63] and (Deltocephalinae + ((Cicadellinae + Typhlocybinae) + (Coelidiinae + (Eurymelinae + Megophthalminae + (Smiliinae + (Darthulinae + Centrotinae)))))) [64].



Figure 4. Cont.



Figure 4. Predicted secondary structure of tRNA genes in the S. shinshana and S. bacilla mitogenomes.

The monophyly of two tribes (Typhlocybini and Erythroneurini) was generally well supported in the subfamily Typhlocybinae, which is consistent with the findings of some previous molecular phylogenetic studies [65,66]. Ten species of Typhlocybini and eight species of Erythroneurini are clustered together, respectively, and most phylogenetic relationships demonstrated a higher nodal support in both ML (BS = 100) and BI (PP = 1) analyses. Our results further confirmed that *S. shinshana* is closely related to the genus *Empoascanara*, while *S. bacilla* and the genus *Mitjaevia* have a closer relationship (Figure 5). In terms of morphological identification, it is easier to distinguish *S. shinshana* from other leafhoppers by observing the appearance. Its body is yellow or yellow-green, but the genera *Seriana*, *Mitjaevia* and *Empoascanara* are difficult to distinguish. Thus, dissection of the male genitals is necessary. Combined with the appearance and the shape of genitalia, *S. shinshana*

and *S. bacilla* belong in Erythroneurini (Hemiptera: Cicadellidae: Typhlocybinae). Although the two species belong to the group of Erythronrurini, other species were used to analyze the phylogenetic relationship at the mitochondrial DNA sequence level in order to elucidate their phylogenetic status and verify consistency with traditional taxonomy.



Figure 5. Phylogenetic trees of forty species of Cicadellidae and two groups inferred by maximum likelihood (ML) and Bayesian (BI) methods based on protein-coding genes. The red tick represents the species in this study.

4. Conclusions

Consistent with previous results for other Typhlocybinae species, this study presents the mitogenome sequences of *S. shinshana* and *S. bacilla*, which are highly conserved in gene size and organization, highly A+T-biased base composition, codon usage of protein-coding genes and secondary structures of tRNAs. In addition, there are no gene rearrangements. This work provides the basic information to perform comparative analyses and discussion of the mitogenome evolution of Erythroneurini. Phylogenetic analyses support all sub-families as monophyletic groups and *S. shinshana* and *S. bacilla* as part of Erythroneurini. However, larger scale studies with more taxa are still needed to enrich the mitochondrial genome database and construct more comprehensive phylogenies to support the results of our study. Our study offers valuable data and an efficient framework for the future phylogenetic research of Typhlocybinae.

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